Expression of Tissue Markers in Malignant and Benign Melanocytic Proliferations – a Comparative Study

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Abstract

Background The most efficient treatment for cutaneous melanoma is surgical excision in its early stage, which involves an accurate differentiation between melanoma and atypical nevi.

Objectives The aim of this study was to investigate the expression of GDF15, CYR61, KRT18 and BCL-2 in cutaneous melanoma, atypical nevi and common nevi and to assess their possible role in differentiating malignant and benign lesions.

Material and Methods Tissues samples from 13 invasive cutaneous melanomas, 11 dysplastic nevi and 14 common nevi as well as ten normal skin tissue samples were used for gene expression analysis. Light Cycler 480 (Roche Technologies) and ΔΔCt method was used to evaluate the expression of genes of interest.

Results Gene CYR 61 proved to be down-regulated in cutaneous melanoma and common melanocytic nevi and up-regulated in dysplastic melanocytic nevi. GDF15 expression is progressively up-regulated from common nevi to dysplastic nevi and melanoma. KRT18 is down-regulated in common nevi and melanoma, and is slightly up-regulated in dysplastic nevi. BCL2 expression is progressively down-regulated from common nevi to dysplastic nevi and melanoma.

Conclusions GDF15 could represent an important factor for melanoma progression and for differential diagnosis. CYR61 seems to be an early marker of neoplasia, but its capacity to distinguish benign lesions from early malignant ones has to be tested on larger studies that also include in situ lesions. BCL2 does not seem to specifically contribute to differentiating benign lesions from malignant ones. KRT 18 could play a role in cell proliferation and distinguishing typical lesions from dysplastic ones.

Key words: melanocytic lesions, CYR61, GDF15, KRT18, BCL2

1. Introduction

Cutaneous melanoma represents a very aggressive type of cancer that can metastasize in the early stages of its progression. Melanoma has one of the fastest growing incidences worldwide, with over 150000 new cases estimated in developing countries in 2010. Although it represents only 10% of the total cutaneous malignant tumours, melanoma is responsible for over 90% of the deaths caused by these tumours.
Currently, the most efficient treatment for cutaneous melanoma is surgical excision in its early stage, which involves an accurate differentiation between melanoma and atypical nevi. Unlike melanoma, which has a very fast proliferation rate, dysplastic nevi lack the uncontrolled proliferation capacity and do not metastasize. Previous studies support the concept that primary and metastatic melanomas are characterized by distinct gene expression patterns when compared to benign melanocytic nevi. However, new markers need to be evaluated further in order to accurately recognize malignant melanocytic proliferations.

The aim of this study was to investigate the expression of GDF15, CYR61, KRT18 and BCL2, involved in the modulation of angiogenesis, cell proliferation and apoptosis in malignant and benign melanocytic lesions and to assess their possible role in differentiating these lesions, considering that angiogenesis, cell proliferation and apoptosis are essential for tumour growth, invasion and metastasis.

2. Materials and method

Biological samples

Thirty eight biopsy specimens selected from patients with melanocytic lesions harvested in the Department of Dermatology from Cluj-Napoca, Romania were considered for this study. The sample series included 13 invasive cutaneous melanomas (CM), 11 atypical nevi (AN) and 14 common nevi (CN). Ten normal skin samples were considered as normal control (NT). All patients signed an informed consent. Tissue samples were immediately frozen and stored in liquid nitrogen until their processing. The standard histopathologic diagnosis was performed, study fragments being collected from dermatoscopically regular and homogenous areas. Small-sized lesions were excluded from the study considering that istopathological diagnosis would have been influenced by research tissue sampling in their cases. The present study was approved by the IRB of the Iuliu Hatieganu University of Medicine and Pharmacy from Cluj-Napoca, Romania.

Total RNA extraction and purification

Total RNA was extracted with TriReagent and purified with RNeasy Mini kit (Qiagen) based on column purification. All extracted RNAs were verified for quality and quantity control with Bioanalyzer 2100 and Nanodrop ND-1000). RNAs with RIN (RNA Integrity Number) >8 were considered for future analysis.

Quantitative PCR reaction (qRT-PCR)

Roche technology (Light Cycler 480) using specific primers and UPL (Universal Probe Library) probes was used. Five hundred nanograms of RNA was used for reverse transcription using First Strand cDNA Synthesis Kit (Roche) kit.

The diluted cDNA (1:10) was then amplified using Light Cycler Taqman Master Kit (Roche). Specific primers and UPL probes for the target genes were established through in silico studies (Roche Applied Science software) as follows: CYR61 (NM_001554.4): F-aagaaacccggatttgtgag, R-gctgcatttcttgcccttt, (upl#66); GDF15 (NM_004864.2): F-ccggatactcacgccaga, R-agagatacgcaggtgcaggt (UPL #28); KRT18 (NM_000224.2): F-tgatgacaccaatatcacacga, R-ctgggcttgtaggcctttta, (UPL#63); BCL2 (NM-000633.2): F- agtacctgaaccggcacct, R-gccgtacagttccacaaagg, (UPL#75) and 18S (NR_003286.2): F-gcaatatctccacatcagga, R-gggacttaatcaacgcacgc (UPL#48). Thermal cycling conditions included: enzyme activation at 95°C for 10 minutes; 11366
PCR amplification (40 cycles): denaturation for 15 s at 95°C, annealing for 20s at 55°C and amplification for 1 s at 72°C; ending of PCR reaction by cooling for 30 s at 40°C. Fluorescence signal was registered in every step of PCR amplification with the setting specific for UPL probes.

Fluorescence signals with Cₚ values> 35 cycles, considered false positive results, were not considered for analysis. The expression of every gene was calculated using ΔΔCₚ method⁶, the Cₚ values being previously normalized to the Cₚ values of the housekeeping gene (18S). Gene expression in melanocytic nevi, dysplastic nevi and melanomas was compared to average expression of each gene in normal tissue as reference.

Statistical analysis

Statistical analysis was made in SPSS software version 16. Shapiro-Wilks test and numerical methods (skewness and kurtosis indices) were used to evaluate the normality of data. Homogeneity of variance was tested with Levene’s test. The differences between groups were assessed by one-way ANOVA of the log-transformed data, followed by Bonferroni test in the case of a homogenous distribution and Tamhane test in the case of a nonhomogeneous distribution. The significance threshold for all of the tests was p=0.05.

3. Results

Fold regulation (FR) values for each gene in every group were calculated relative to the control group and all data are presented as mean ± standard error of mean (SEM). The genes with FR ≥±1.5 and p < 0.05 were considered significantly differentially expressed.

We found highly up-regulated levels of GDF15 in cutaneous melanoma compared to normal skin (FR=17.09, p=0.033) and common nevi (FR=13.89, p=0.033). Significantly increased expression of GDF15 was also observed in dysplastic nevi compared to normal skin (FR=3.73, p=0.012) and common nevi (FR=3.03, p=0.02) (Fig. 1). There were no significant differences in cutaneous melanoma compared to dysplastic nevi, although we observed an over expression of GDF15 in cutaneous melanoma (FR=4.58) (Fig. 1).

Figure 1. Mean values (± SEM) of GDF15 expression levels within the study groups (* p <0.05). FR value was calculated against the mean value of the control group (NT).

Figure 2. Mean values (± SEM) of CYR61 expression levels within the study groups (***p<0.001). FR value was calculated against the mean value of the control group (NT).
For CYR61, the only significant differences were observed in dysplastic nevi compared to common nevi (FR = 6.42, p<0.001). However, we also observed an under expression of CYR61 in melanoma (FR = -1.5) and common nevi (FR = -3.85) compared to normal tissue, as well as an overexpression in dysplastic nevi compared to normal tissue (FR = 1.67) (Fig. 2).

Our results highlighted a significant overexpression of BCL2 in common nevi compared to cutaneous melanoma (FR = 1.84, p = 0.042) and normal skin (FR = 2.91, p = 0.01) (Fig. 3).

KRT18 was significantly down-regulated in melanoma (FR = -2.44, p = 0.038) and common nevi (FR = -11.11, p < 0.001) compared to normal tissue, but no significant expression changes were observed in dysplastic nevi relative to normal skin (FR = 1.42, p = 0.9) (Fig. 2). We also observed a highly increased expression of KRT18 in dysplastic nevi compared to common nevi (FR = 15.78, p = 0.001) (Fig. 4).

4. Discussions

In this study, we evaluated the expression of four genes: GFD15, CYR61, KRT18, BCL2, involved in modulation of angiogenesis, cell proliferation and apoptosis in malignant and benign melanocytic lesions as well as in normal skin.

GDF15 is a member of the TGF-β family, whose expression increases during inflammation, cancer and metabolism\(^7\). The role played by GDF15 in the development and progression of cancer is complex and not fully understood\(^8\). Some experimental evidence suggests that GDF15 has tumour suppressor activity\(^9,10\), while other data suggests that it has oncogenic activity\(^11\). In our study we found high levels of GDF15 in cutaneous melanomas compared to dysplastic nevi, common nevi and normal skin. There are similarities between our findings and those reported by Mauerer et al.\(^12\), which described a significant up-regulation of GDF15 in primary and metastatic melanoma compared to melanocytic nevi. Our results highlight the role of GDF15 for melanoma progression, strengthening the remark of Talantov et al.\(^13\) who suggested that GDF15 could better differentiate between melanoma and melanocytic nevi in comparison to more conventional markers (e.g., MART1, tyrosinase, me20m).
We also chose to evaluate CYR61 as a possible marker for melanoma diagnosis, because it plays multiple roles in cancer progression including modulation of cell proliferation, differentiation, angiogenesis, and apoptosis. CYR61 is a member of the CCN family (growth factor-inducible immediate-early gene family) whose expression is enhanced by MAPK. There are a few studies describing the role of CYR61 in melanoma progression but previous reports showed an up-regulation of CYR61 in dysplastic or in situ malignant lesions, located at gastric and cervical level, as well as a down-regulation in invasive malignant lesions compared with normal cells, indicating its role in tumor suppression in early-stage lesions as a response to stress. This is in accordance with our data, as we report an up-regulation of CYR61 in dysplastic nevi compared to common nevi and melanoma. In light of the up-regulation of this gene in dysplastic nevi and its down-regulation in invasive melanoma and common nevi observed in our study, it seems that CYR61 expression follows the same pattern in melanoma progression and could thus be considered an early marker of neoplasia. This hypothesis must be tested on larger samples that include also in situ melanomas.

BCL2, the third molecule evaluated in our study, represents one of the most important pro-survival molecule, which can protect melanoma cells from apoptosis. The role of proteins within the BCL2 family has been extensively studied in melanocytic tumors and the present results are in line with previous studies. These demonstrated an up-regulation of BCL2 in nevi as compared to normal skin and a down-regulation in primary and then metastatic melanoma. We found statistically significant difference between common nevi and cutaneous melanoma, on the one hand, and normal skin, on the other hand. Therefore, BCL2 level places dysplastic nevi between common nevi and melanoma but does not enable a differential diagnosis of melanocytic lesions.

Keratins are important protectors of epithelial structural integrity under conditions of stress, but have also been recognized as regulators of other cellular functions, including motility, signaling, growth and protein synthesis. Although melanoma is considered negative to immunohistochemical assay of keratins, this being the particular characteristic that distinguishes it from poorly differentiated carcinomas, there are studies that identified keratins, and especially KRT18 expression in melanocytic cell cultures or melanomas. To our knowledge, KRT18 expression in melanocytic nevi has not been previously studied. Our study reported a down-regulation of this gene in melanoma and common nevi compared to normal skin, and a minimal up-regulation in dysplastic nevi. Comparing KRT18 within the group of melanocytic tumors, the highest up-regulation was observed in dysplastic nevi, followed by melanoma and common nevi. A previous study in an animal model identified an up-regulation of KRT8/18 complex at hepatic level, in pre-malignant or dysplastic lesions compared to normal hepatocytes, demonstrating the cell proliferative effect of this complex. Further research is needed to assess the exact role of KRT18 in melanocytic proliferations and to confirm its capacity to distinguish melanocytic lesions.

In summary, CYR61 seems to be an early marker of neoplasia, but its capacity to distinguish benign lesions from early malignant ones has to be tested on larger samples that also include in situ lesions. GDF15 differentiates between melanocytic lesions considering that it is gradually up-regulated as normal melanocytes progress to melanoma. BCL2 does not seem to specifically contribute to differentiating benign lesions from malignant ones. KRT 18 could play a role in cell proliferation and distinguishing typical lesions from dysplastic ones. We believe that GDF15 and CYR61 represent two important factors of melanoma progression related to hypoxia, but this hypothesis must be tested on larger samples.
Our study is limited by the small number of investigated cases. Consequently, further validation is needed, on larger batches. Moreover, to ensure that the genes we found to be differentially expressed in our study are useful for differential diagnosis, further studies are necessary, especially investigations (e.g. immunohistochemical analysis) to determine if gene expression is translated into protein expression. In spite of these drawbacks, this study can be seen as bringing new and relevant information to the so much needed study of molecular markers in melanocytic lesions.

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